

CAN MINOCYCLINE REDUCE CYTOKINE-INDUCED BONE RESORPTION AFTER SCI?

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ABSTRACT

Can Minocycline Reduce Cytokine-Induced Bone Resorption After SCI? (May 2016)

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Spinal contusion injuries are characterized by an increased excitation of the immune system that results in inflammation and heightened expression levels of pro-inflammatory cytokines in the central nervous system (CNS) (Donnelly et al. 2008). These inflammatory cytokines impair bone homeostasis to favor bone resorption over the secretion of bone matrix, thus contributing to an overall decline in bone mineral density (BMD) (Perez-Castrillon et al. 2000, Schett 2011). Maintenance of bone integrity following spinal cord injury (SCI) is critical because low BMD compromises locomotor and sensory recovery, and is a predictor of bone pain, future fractures, and osteoporosis. We proposed that minocycline, an FDA-approved anti-inflammatory drug (Kobayashi et al. 2013), may protect against the BMD deterioration routinely observed after SCI. To test this, male Sprague Dawley rats were given a moderate spinal contusion injury and then treated with 0 or 0.33 mg/kg/ml of minocycline from days 1-14 following injury. Recovery of function was assessed for 28 days post injury. At the end of the experiment, bone samples were collected to assess BMD and changes in amount of mineralization. Administration of minocycline significantly enhanced active bone mineralization and overall new bone formation rates in a rodent SCI model compared to vehicle-treated SCI controls. A more comprehensive

understanding of the effects of different therapeutic strategies for alleviating post-injury bone loss is important for the development of effective treatment strategies to improve long-term rehabilitation following SCI.

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CHAPTER I

INTRODUCTION

The structure of bone is a unique composite of living cells embedded in a three-dimensional mineralized structure of inorganic salts and organic matrix. The inorganic salt component of bone primarily consists of phosphate and calcium ions, but also includes substantial amounts of sodium, potassium, magnesium, carbonate, fluorite, zinc, barium, strontium, and other minerals. The organic matrix of bone consists of collagenous proteins and non-collagenous proteins such as bone morphogenetic proteins (BMPs), osteocalcin, fibronectin, osteonectin, and growth factors (Boskey et al. 2002). The extracellular matrix of bone is a complex and organized framework that gives it mechanical support and serves as a substrate for bone homeostasis. Like most dynamic tissues, bone has the ability to adapt to its environment and it does so by changing its internal structure through the resorption of old bone and the laying down of new bone matrix, a process collectively called bone remodeling. Remodeling is a fundamental characteristic of bone that helps it manage the delicate equilibrium between mineral resorption and deposition to ensure skeletal integrity. When the balance between bone formation and resorption is lost in favor of the latter, the consequence is bone loss and a number of bone diseases. To illustrate the effects of a disruption in the equilibrium between formation and resorption in bone homeostasis, the mechanisms behind periodontitis and osteopetrosis may be considered. In periodontitis, a bacterial infection induces the proliferation of inflammatory cells that produce cytokines, or chemical mediators, that stimulate the accumulation of osteoclasts. This abundance of osteoclasts results in abnormally increased bone resorption in the alveolar bone to produce loosening of

teeth. In contrast, those suffering with osteopetrosis have mutated osteoclasts with diminished resorptive functions. The consequence of decreased bone resorption from these mutated osteoclasts is the excessive accumulation of bone mass which, like reduced BMD, also results in an increased likelihood of fractures (Longhini et al. 2014, Sobacchi et al. 2013). Because the remodeling process is so intimately tied to the pathophysiology of osteoporotic and osteopetrotic conditions, understanding how remodeling is regulated is key to the effective prevention of bone-related afflictions. The coordinated activities of two types of cells determine the outcome of bone metabolism: osteoblasts that secrete matrix for bone formation and osteoclasts that absorb bone tissue during growth and healing. Osteoblasts are of mesenchymal origin while osteoclasts are hematopoietic in origin and are derived from the monocyte phagocytic system of organisms (Gilbert 2000).

Processes of bone remodeling

Two processes result in the formation of healthy bone tissue: intramembranous ossification and endochondral ossification. The former occurs when bone is directly laid down into primitive connective tissue called mesenchyme while the latter occurs when cartilage is utilized as a precursor to the bone. In intramembranous ossification, osteoblasts secrete a collagen-proteoglycan matrix that binds calcium salts to produce calcified bone. Osteoblasts are typically kept separate from the site of calcification when collagen-proteoglycan matrix is being bound to osteoid or pre-bone matrix, but periodically osteoblasts may become trapped in the calcified matrix and become osteocytes, or bone cells. As calcification progresses, bone spicules emit from the region where ossification began and the entire locale of spicules radiation eventually

becomes encompassed by compact mesenchymal cells to form the periosteum, a layer of vascular connective tissue that envelopes the bone. Cells on the internal surface of the periosteum differentiate into osteoblasts and continue to deposit osteoid matrix parallel to the existing aforementioned spicules to form the many layers of bone. The mechanism behind intramembranous ossification involves bone morphogenetic proteins (BMPs) and a transcription factor, CBFA1, that activates the genes for a multitude of bone-specific extracellular matrix proteins. Mesenchymal cells aggregate to form the cartilage precursor to be replaced with bone in endochondral ossification, which can be organized into five phases. During the first phase, a portion of mesenchymal cells are selected to differentiate into cartilage cells, which is facilitated by paracrine factors that induce nearby mesodermal cells to express transcription factors, Pax1 and Scleraxis, that activate cartilage-specific genes and induce the formation of cartilaginous precursors to bone. In the course of the second phase, the selected mesenchymal cells condense and differentiate into chondrocytes, or cartilage cells. In the third phase, the chondrocytes divide rapidly and secrete a cartilage-specific extracellular matrix that becomes the framework of the premature bone. In the fourth phase, the chondrocytes cease division and become enlarged, or hypertrophic, chondrocytes that have the ability to alter the cartilaginous matrix with the addition of collagen X and excess fibronectin. This alteration allows the cartilage to be mineralized with calcium carbonate. Hypertrophic chondrocytes also release small membrane-bound vesicles containing enzymes active in the generation of calcium and initiation of mineralization into the cartilage-specific extracellular matrix. The fifth and final phase of endochondral ossification entails the apoptosis of the hypertrophic chondrocytes and the annexation of the completed cartilage substructure with blood vessels. The space left behind by the enlarged chondrocytes

become bone marrow and cells in its vicinity differentiate into osteoblasts that proceed to synthesize bone matrix on the surface of the cartilage framework. The cartilage precursor is simultaneously degraded as osteoblasts work to lay down new bone matrix in its place, thus endochondral ossification is characterized by the use of cartilage as a blueprint for the final bone product. This replacement of cartilage cells by bone cells is contingent upon the mineralization of the cartilage-specific extracellular matrix with calcium carbonate and the hypertrophy of chondrocytes (Gilbert 2000).

While synthesis of bone is done by osteoblasts, the destruction of bone tissue is the work of osteoclasts, which are terminally differentiated multinucleated cells that access bone through the blood vessels previously described in the endochondral process of bone formation (Gilbert 2000). Aside from osteoclasts, blood vessels also import the blood-forming cells of bone marrow. To elaborate on its origins, osteoclasts are thought to be derived from the same progenitor cells as macrophage blood cells and have the ability to dissolve both inorganic and protein constituents of bone matrix (Florencio-Silva et al. 2015). The cell membranes of osteoclasts are endowed with energetic ion channels that pump protons into their extracellular space to cause a drastic lowering of pH within the cells' own microenvironment. Further, osteoclasts bear many vesicles and vacuoles containing lysosomes filled with acid phosphatase and collagenase that work together to dissolve neighboring bone mineral. Numerous cellular processes radiate into bone matrix from osteoclasts to pump hydrogen ions out and acidify tissue in order to effectively solubilize it (Kornak et al. 2001, Graves et al. 2008). Due to its catabolic nature, osteoclast numbers must be rigorously regulated to ensure coherence of bone. At a site of active bone

resorption, the osteoclast forms a specialized extensively folded cell membrane that facilitates bone removal by increasing the cell surface for the secretion of proteins that catabolize bone and for the uptake of resorption contents (Lin et al. 2015). During remodeling, osteoclasts divide to produce four types of osteoclastic membrane domains: the sealing zone, the ruffled border, the basolateral secretory domain, and the functional secretory domains. The former two are kept in contact with the bone matrix while the latter two are not. The ruffled border is fabricated by microvilli isolated from adjoining tissue by the sealing zone, which is an area deprived of organelles near the edges of the osteoclast adjacent to the bone matrix (Gilbert 2000). The ruffled border contains a hydrogen ion ATPase that helps to acidify resorption lacunae to enable the dissolution of bone mineral. Because the ruffled border is formed due to acute trafficking of lysosomal and endosomal components, its maintenance is essential for osteoclast activity. The products of this degradation of extracellular matrix are eventually endocytosed across the ruffled border and transcytosed to the functional secretory domain located at the plasma membrane. There is evidence that osteoclasts exhibit several other functions in addition to bone mineral dissolution. Some findings have shown that osteoclasts have the ability to release clastokines, or osteoclast-derived coupling factors, that regulate osteoblast activity during bone remodeling (Schell et al. 2006). Here, coupling refers to the collaborative processes of bone formation and bone resorption. Other findings indicate that osteoclasts may play a role in the regulation of the hematopoietic stem cell niche (Mansour et al. 2012). Together, these findings suggest that osteoclasts are a source of cytokines that influence the activity of other cells.

Role of cytokines in bone remodeling

The activity of both osteoblasts and osteoclasts are regulated by numerous factors, one of them being cytokines. Cytokines are regulatory factors secreted by cells to manage host immune responses to infection, trauma, and inflammation. Cytokines can worsen conditions (pro-inflammatory) or promote healing (anti-inflammatory), but are often detrimental to the host during incidents of overwhelming trauma. Interleukin (IL-1) and tumor necrosis factor (TNF) are examples of pro-inflammatory cytokines that can result in fever, inflammation, and necrotic tissue. There is emerging evidence to suggest that cytokines determine whether mesenchymal cells differentiate into osteoclasts or other bone-related cell lineages. Specifically, studies have shown that pro-inflammatory cytokines have the potential to stimulate osteoclast formation, consequently increasing bone resorption (Weitzmann 2013). In osteoclastogenesis, the stimulation of mononuclear osteoclast progenitors by macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL) is crucial. Both M-CSF and RANKL serve as inducers of osteoclast differentiation and promoters of mononuclear progenitor fusion to mature, multinucleated osteoclasts. M-CSF binds to its receptor, cFMS, in osteoclast precursors to stimulate their proliferation and inhibit their apoptosis. When RANKL binds to its receptor, RANK, in osteoclast precursors, osteoclast proliferation is likewise stimulated. Osteoprotegerin (OPG) is the third component of the RANKL/RANK mechanism that serves as a decoy receptor for RANKL. OPG binds to RANKL and prevents it from associating with its customary receptor, RANK, to inhibit osteoclast differentiation. Numerous other inflammatory cytokines similar to RANKL are able to drive osteoclastogenesis and regulate bone resorptive activities (Weitzmann 2013).

SCI, cytokines, and bone remodeling

Concentrations of TNF, a cell death-inducing cytokine, increases after SCI but intraperitoneal injections of the anti-inflammatory cytokine interleukin-10 (IL-10) half an hour post-injury were able to mitigate the production of TNF- α by macrophages. Additionally, the same IL-10 injections also improved recovery of hindlimb function as measured by the BBB locomotor rating scale. Not only do the outcomes of this study insinuate that early attenuation of inflammation may be beneficial for neuroprotection, but they also insinuate a relationship concerning the regulation of pro-inflammatory cytokines with anti-inflammatory factors, TNF- α and IL-10 in this case, respectively. It seems that a reduction in inflammation is sufficient for substantial improvement in locomotor function post-SCI. The question now is whether this minimization of inflammation by means of anti-inflammatory factors is also sufficient for improvement in bone preservation and recovery, seeing that recovery of locomotor function is often associated with a degree of bone integrity. This study provides significant implications for conditions characterized by inflammation near bone, such as SCI, because it suggests a direct relationship between inflammation and skeletal integrity. Following injury to the spinal cord, inflammatory reactions take place that result in increased pro-inflammatory cytokine levels in serum, the clear liquid partitioned from blood coagulate (Brewer et al. 1999). This inflammation may impact bone formation. Indeed, studies examining the effects of SCI on bone mineral density (BMD), a measure reflecting the strength of bones by calcium content, have shown decreased mineral values in measured regions of the lumbar spine and hip compared to that of controls. Specifically, BMD values of the hip decreased as the duration of SCI increased and

levels of calcium in 24-hour urine samples were found to be significantly higher in the patient group than the control group (Kurtulus et al. 2006). SCI-induced bone loss portrays serious osteoporosis with no effective treatment.

In addition to SCI per se, medications that are routinely used to treat pain, including opiates such as morphine, may affect skeletal integrity. Morphine significantly increases levels of pro-inflammatory cytokines, specifically IL-1 β , after SCI (Hook et al. 2011). Studies have also shown that 50% of a cohort receiving intrathecal opioid doses for SCI-related pain demonstrated osteopenia, with more than 20% of the cases diagnosed as osteoporosis, the more severe form of bone loss (Duarte et al. 2013). Opioids are thought to impede bone synthesis by binding to mu receptors located on osteoblasts and directly inhibiting their activity. Osteoblast inhibition leads to decreased synthesis of bone, and subsequently decreased BMD. Osteocalcin, a non-collagenous protein of bone, can be utilized as a biomarker for osteoblast activity and chronic opioids users have been shown to exhibit decreased osteocalcin concentrations (Shadid et al. 2013). In addition to their inhibitory effects on osteoblasts, opioid use can also engender osteoporosis by suppressing the production of certain hormones like testosterone, which leads to a condition called hypogonadism, which can weaken bones in both men and women (Brennan 2013, Smith et al. 2012). Conventional analgesics, including opioids, are among the most effective treatments for pain. Alarming, these studies suggest that opioids significantly undermine the long-term recovery of bone mineral and counter-intuitively increase the expression of symptoms of neuropathic pain in a rodent model of SCI (Hook et al. 2011). This

calls for improved therapies for bone-related traumas that accounts not only for the onset of neuropathic pain, but for prevention of bone loss as well.

Investigating the relationship between bone remodeling and inflammation after SCI

The inflammatory response exhibited by the immune system is a result of tissue injury; damaged cells release chemicals that promote swelling. The role of inflammation in the midst of SCI is evidently beneficial for neural recovery, but it also bears deleterious effects. There is evidence that immediate inflammatory responses in the CNS are specialized to curb possible infection and clean up cellular debris rather than to aid in the post-trauma repair processes (Zhang 2009). Injury activates the transformation of quiescent microglia into macrophage-like cells that produce pro-inflammatory cytokines. These pro-inflammatory cytokines and other analogous cellular factors spark a response cascade that recruits additional quiescent microglia. Further, the presence of cellular debris stimulates dormant astrocytes to express cytokines, glial fibrillary acidic protein (GFAP) and various other factors that may contribute to prolonged inflammation (Watkins 2002). Since molecules typically involved in immune function are present at the site of SCI, this is an ideal research model in which to investigate possible pharmacological interventions that may counter the deleterious effects of molecular immune responses on bone density.

Minocycline is a broad-spectrum tetracycline antibiotic, meaning that it is a general-use bacterial inhibitor. Of the tetracycline-class antibiotics, minocycline is the most lipid-soluble and is able to penetrate into the brain with relative ease. In previous rodent SCI models, intravenous

administration of minocycline improved neurological outcomes, reduced neuronal apoptosis, decreased microglial activation, and reduced inflammation (Wells 2003). Minocycline is currently in phase III clinical trials, meaning it is being given to large groups of people to confirm its effectiveness, monitor its side effects, compare it to commonly used treatments, and collect information that will enable the pharmaceutical to be safely utilized (<https://clinicaltrials.gov/ct2/show/NCT01828203>). Due to the link between pro-inflammatory cytokines and bone disease, minocycline, an antibiotic glial inhibitor that has been shown to reduce inflammation in vivo (Kobayashi 2013), may be able to counter the deleterious effects of post-trauma immune responses on bone homeostasis and improve mineral retention in a rodent model of spinal cord injury. The present experiment will test this hypothesis and will further understanding of the correlation between bone integrity and inflammation. Moreover, this study will inform clinical practice by evaluating therapeutic strategies that may reduce osteoporotic comorbidity in individuals with SCI and improve long-term recovery. Low BMD and bone disease are serious afflictions that may hinder the holistic recovery of SCI patients not just physically, but mentally as well due to excessive amounts of neuropathic pain (Nicholson et al. 2004). Further, bone retention is also crucial for other injuries in proximity to bone aside from SCI because they tend to bear similar models of recovery with analogous issues. Consequently, there should be an urgency to alleviate these sufferings not only for the benefit of the hundreds of thousands that experience SCI every year but for those experiencing other bone-related injuries such as periodontitis, arthritis, and various others as well.

CHAPTER II

METHODS

Twelve singly-housed male Sprague-Dawley rats were given a moderate spinal contusion injury (IH impactor, 150 kdynes with 1 second dwell time) at the T12 vertebral level using the Infinite Horizon spinal cord impactor (PSI, Fairfax Station, VA). Subjects were anesthetized with isoflurane (5%, gas), and after stable anesthesia was achieved the concentration of isoflurane was lowered to 2-3%. Twenty-four hours after injury, and after balancing locomotor function (BBB scores) across groups, the SCI subjects were randomly assigned to one of two treatment conditions: 0 or 0.33 mg/kg/ml minocycline. They were provided with free access to the minocycline in their drinking bottles from days 1-14 post-injury. The daily amount of fluid/minocycline consumed was recorded. To control for the effects of SCI *per se* on bone loss, locomotor recovery and osteological assessments were also conducted with eight intact rats.

Locomotor recovery assessments

Recovery was assessed for a 28-day period following SCI. Locomotor recovery was determined using the BBB scale (Basso et al. 1995) in an open enclosure (99 cm diameter, 23 cm deep). The converted BBB scale (Ferguson et al. 2004) is a 12-point scale that is conventionally used as an index of hindlimb functioning after a spinal injury. Using this scale, no movement of the hindlimbs (ankle, knee or hip) is designated a score of 0, and intermediate milestones include slight movement of one joint (1), extensive movement of all three joints (5), occasional weight supported stepping in the absence of coordination (8), and consistent weight supported stepping

with consistent FL-HL coordination (12). Baseline motor function was assessed on the day following injury and prior to drug treatment. BBB scores were also collected on Days 1-7, 9, 11, 13, 15, 18, 21, 24, and 28 post injury. Because rodents often freeze when first introduced to a new apparatus, subjects were acclimated to the observation fields for 5 min per day for 3 days prior to surgery. Each subject was placed in the open field and observed for 4 min. Care was taken to ensure that the investigators' scoring behavior had high intra- and inter-observer reliability (all r 's > 0.89) and that they were blind to the subject's experimental treatment.

At the end of the 28-day assessment period, subjects received a lethal injection of beuthanasia (100 mg/kg) and the injured spinal (1 cm section centered on the lesion) and bone tissue was collected and processed to assess changes in bone formation with minocycline treatment. This two dose experimental design required twelve rats. Eight additional intact subjects were assessed as injury controls.

Osteological assessments

Bone analyses were conducted using dynamic cortical histomorphometric methods (Dempster et al. 2013) and peripheral quantitative computed tomography (pQCT). Subcutaneous injections of calcein, an orange-colored fluorescent dye, were administered on days 21 and 26 post-injury (7 and 2 days prior to termination) to label mineralizing surfaces for later determination of bone formation rates. Un-demineralized, excised left distal tibias were fixed in 4% phosphate-buffered formalin for 24 hours, and then serially dehydrated and embedded in methyl-methacrylate. Serial cross sections (approximately 100 μ m thick) of the bone closest to the mid shaft were made on a

diamond wafer IsoMet Low Speed Saw (Buchler, Lake Bluff, IL) starting 1 mm proximal to the tibia-fibular junction. Cross sections of each specimen were analyzed at 20x magnification using OsteoMeasure Analysis Software version 3.3 (OsteoMetrics, Inc., Atlanta, GA). Mineralizing surface to bone surface ratio (MS/BS), mineral apposition rate (MAR), and bone formation rate ($BFR = MS/BS \times MAR$), were determined. MAR is the rate at which new bone is deposited in the radial direction while BFR is an overall measure of new bone formation calculated by multiplying the percentage of surface actively forming new bone with the radial rate of that formation.

Right tibias were similarly preserved in phosphate buffered saline in -35°C. Once thawed, ex-vivo pQCT scans of the proximal tibia metaphysis (mixed cortical and cancellous bone site) and mid-shaft tibia (purely cortical bone site) were completed on a Stratec XCT Research-M device (Norland Corp., Fort Atkinson, WI). Metaphysical volumetric BMD was measured at the proximal tibia and distal femur from 4 slices located at least 1 mm distal of the growth plate. Three contiguous slices were averaged to provide one value for each variable at the proximal tibia metaphysis. One mid shaft tibia slice was taken at approximately 50% of the total bone length. Scans were completed at 2.5mm/sec scan speed, 100 μ m voxel resolution, and 0.5 mm slice thickness. Measures obtained from the ex vivo pQCT scans include cortical and total and cortical bone mineral content (BMC). The metaphysis is a broad portion of the length of bone between the epiphysis and diaphysis; it comprises the growth plate, which is the part of the bone that grows during adolescence. Cancellous bone refers to the meshwork of spongy tissue of mature bone typically found at the ends of long bones such as the femur. Cortical bone refers to

the dense outer surface of bone that forms the protective layer around the internal cavity; it constitutes nearly 80% of skeletal mass and is highly resistant to bending and torsion.

CHAPTER III

RESULTS

Locomotor recovery

Mean BBB scores ranged from 6.81 ± 0.56 for the water group and 6.78 ± 0.71 for the minocycline group. A slight increase in locomotor recovery was observed for the minocycline group compared to the water group towards the end of recovery, but there were no significant differences between the two treatment groups (Figure 1).

OsteoMeasure

The mineralizing bone surface to total bone surface ratio (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS) measurements for each SCI treatment group and the intact control group were averaged and the standard errors of the means were calculated. The control group for these three measures consists of 5 rats instead of 8 due to time-related restrictions. Using one-way ANOVAs it was determined that the difference in MAR was not significant between the two SCI treatment groups and the intact group ($F(1, 11)=1.079$, $p>0.05$), but BFR ($F(1, 11)=14.540$, $p<0.0001$) and MS ($F(1, 11)=21.536$, $p<0.0001$) did differ across the groups (Figures 2, 3, and 4, respectively). Duncan's post-hoc showed that BFR was significantly higher in the intact controls, compared to both SCI groups ($p<0.05$). SCI subjects treated with minocycline also had significantly higher BFR than vehicle-treated SCI controls ($p<0.05$). Similarly, MS was significantly higher in the intact subjects, compared to the SCI groups, and higher in minocycline-treated SCI subjects than vehicle-treated SCI controls ($p<0.05$). The BFR

and MS was highest for the intact controls, Minocycline-treated SCI, and then vehicle-treated SCI groups in descending order.

Peripheral quantitative computer tomography

The total bone mineral content (BMC), total bone mineral density (BMD), total bone area, cancellous BMC, cancellous BMD, cortical BMC, and cortical BMD for the rats, including the 8 controls, were assessed using pCQT scanning, averaged for each treatment group, and standard errors of the means were calculated. There was a trend for lower total BMD in the minocycline group compared to the water group (Figure 5), although the difference was not significant ($p>0.05$). There was also a trend for lower total cortical BMD in the minocycline group (Figure 6), although the difference was not significant ($p>0.05$). There were no significant differences in the total cancellous BMD across groups (Figure 7).

Figures

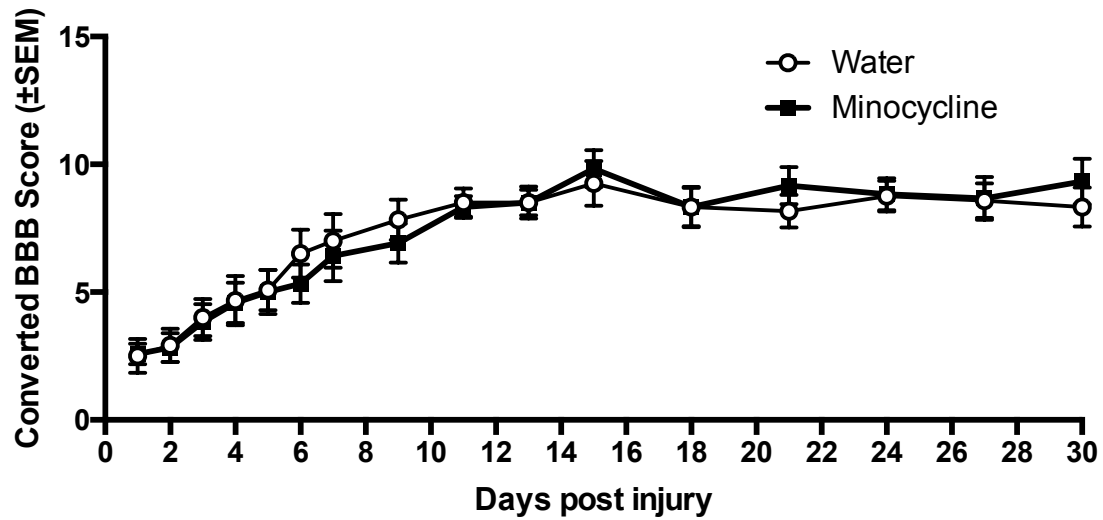


Figure 1. Minocycline treatment did not affect recovery of locomotor function.

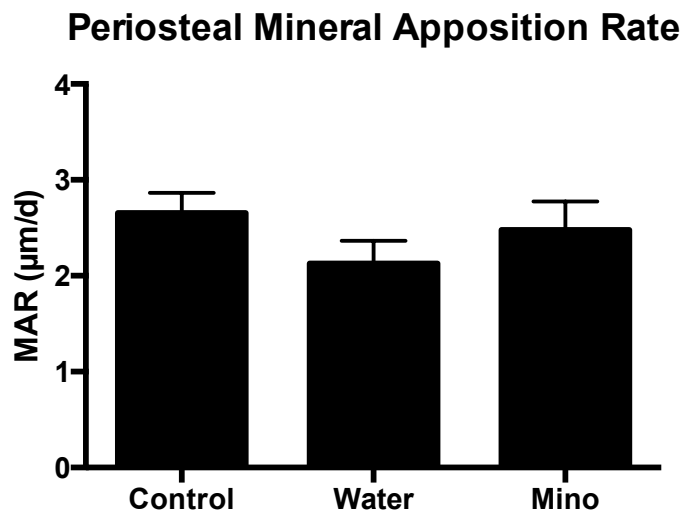


Figure 2. No significant differences in MAR as a result of surgery or drug treatment were observed.

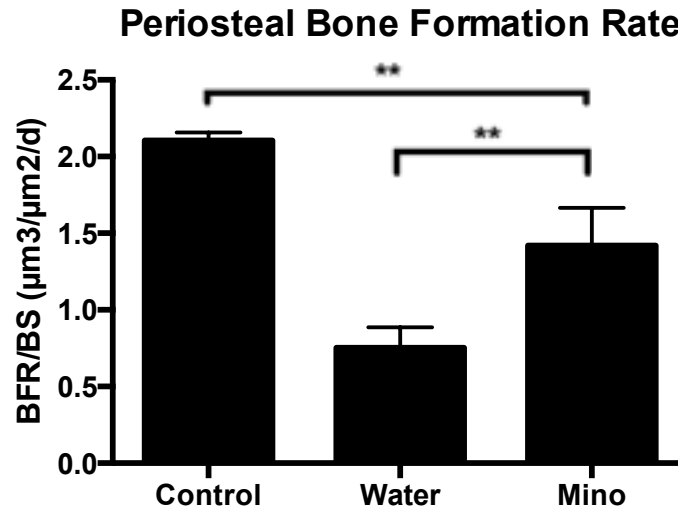


Figure 3. The minocycline group showed significantly higher BFR compared to the water group.
** $p < 0.05$

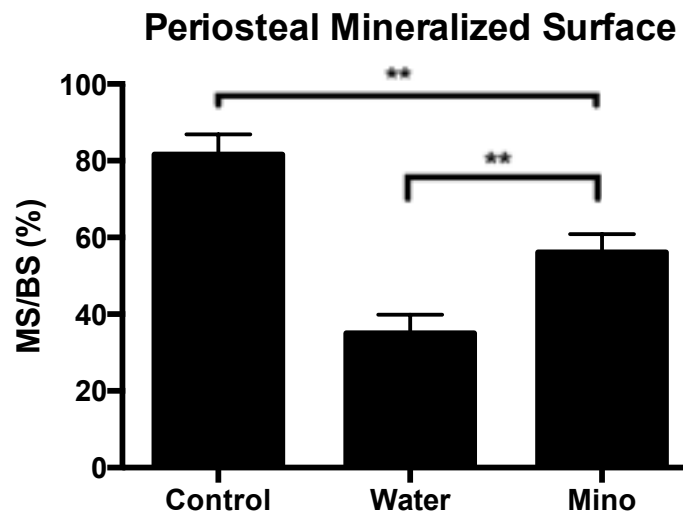


Figure 4. The minocycline group showed significantly higher MS compared to the water group.
** $p < 0.05$

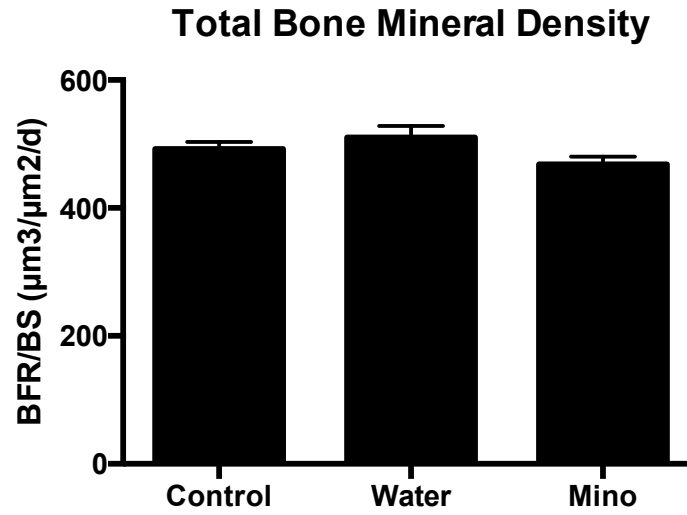


Figure 5. There is a trend for lower total BMD in the minocycline group compared to the vehicle-treated SCI group, though the difference is not significant.

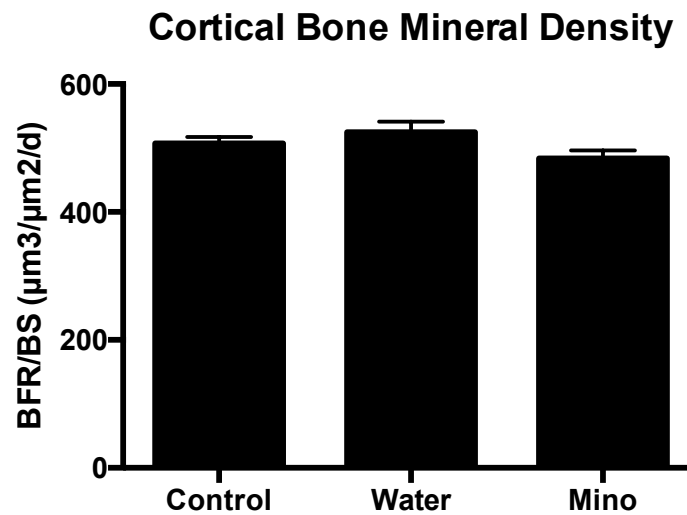


Figure 6. There is a trend for lower total cortical BMD in the minocycline group compared to the vehicle-treated SCI group, though the difference is not significant.

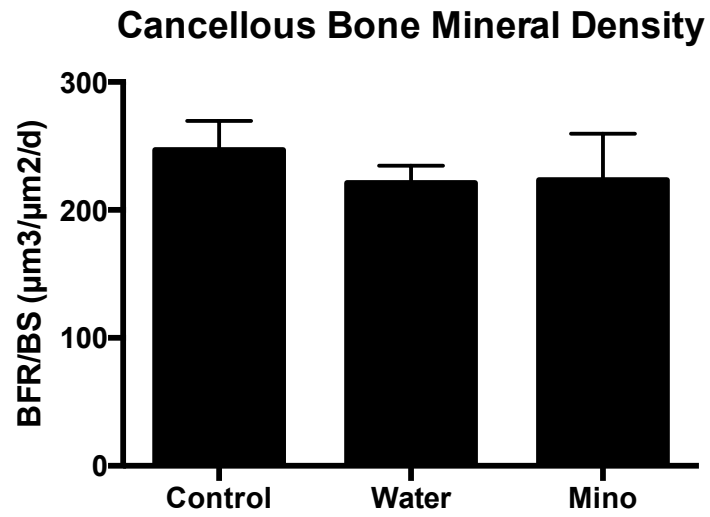


Figure 7. There were no significant differences in total cancellous BMD across surgery of drug treatment groups.

CHAPTER IV

DISCUSSION

Minocycline, an anti-inflammatory glial inhibitor, was able to improve osteological outcomes after a traumatic spinal cord injury. Surprisingly, however, rather than increasing bone density in the rodent model of SCI, minocycline appeared to induce new bone formation. Thus, our initial hypothesis that the tetracycline drug could reduce cytokine-induced bone resorption was not supported (Figure 5). Nonetheless, the drug administration group showed higher levels of mineralizing surface and overall bone formation compared to the water group. This suggests that minocycline may function by increasing osteoblast activity instead of reducing osteoclast activity, which was originally hypothesized. The anti-inflammatory properties of minocycline were thought to counter the consequences of inflammatory cytokines that served to excite osteoclastogenesis, but whether this occurs is not clear with the data provided by this study.

Post-SCI loss of bone mass is typically attributed to an unbalanced bone homeostasis with resorption exceeding bone formation, ultimately leading to the deterioration of bone and the onset of osteoporosis (Jones et al. 2002, Szollar et al. 1998). Further, it has been shown that the duration of paralysis (DoP) is related to the amount of post-SCI bone loss (Dionyssiotis et al. 2011). Due to the loss of muscle activity below the level of injury and the mechanical, neurological, and hormonal changes accompanying SCI, the treatment of bone loss proves to be difficult and research for more effective means to salvage bone is imperative. This rapid decrease in bone density that manifests just days after SCI is reported to last between one to three years

(Garland et al. 1992, Sorensen et al. 1998, Shields 2002). One study observed the rate of bone loss to be 2-4% every month (Wilmet et al. 1995). Another study, following male adults less than 40 years of age with SCI, reported a 33% loss in bone mass of the lower extremities within three to four months post injury, while other studies report as much as 50% loss in BMD within the first three years post injury (Garland et al. 1992, Eser et al. 2004, Sorensen et al. 1998). After the initial episode of rapid bone loss, demineralization decelerates: individuals eventually either reach a steady state or bone will continue to demineralize at a slower rate for several years (Shields 2002, Eser et al. 2004, Rittweger et al. 2006). Bone loss after SCI has been shown to vary between and within regions of the body. Not only do the sublesional and supradesional areas differ in bone metabolism, but also among different bone tissues such as cortical and trabecular (Jones et al. 2002). Specifically, rapid trabecular bone loss has been shown to level off after a few years post SCI while cortical bone has been shown to persist in slowly losing mass for more than ten years (Giangregorio et al. 2006). Trabecular bone is synonymous with cancellous bone, and is abundant in the spine and both proximal and distal epiphysis and metaphysis of long bones. Trabecular bone is more susceptible to demineralization than cortical bone because the remodeling processes of these two types of osseous tissues are markedly distinct (Svircev 2007, Frost 1985). Due to the larger surface area of cancellous bone, more opportunities for metabolic processes are available than in more compact cortical bone surface (Hangartner et al. 1994); one study of bone loss in paraplegic individuals found that cancellous bone loss was twice as fast as cortical bone (Sorensen et al. 1998). In contrast, cortical bone that constitutes much of the diaphysis of long bones undergoes a considerably slower thinning process that gradually reduces overall bone strength (Frey-Rindova et al. 2000). The current study suggests that short-term

minocycline treatment may have a more pronounced effect on spongy trabecular, or cancellous, bone compared to cortical bone (Figures 6 and 7). Since the aforementioned studies exhibited evidence for a greater, more rapid loss in spongy bone than compact bone in the early phase of injury, minocycline bears potential as a therapeutic agent in alleviating post-SCI osteoporosis by bolstering the integrity of trabeculae.

Significant differences in MS and BFR were observed between the minocycline-treated and vehicle-treated SCI groups. MS is a kinetic measure of the extent of bone surface actively mineralizing. BFR takes into account how much of the bone surface is actively mineralizing, which depends on the number of osteoblasts that are active; BFR multiplies the average work of each osteoblast by the fraction of bone surface with active osteoblasts ($MAR \times MS/BS$). MAR is the rate at which osteoblasts are making matrix, and since matrix calcifies at a constant rate, MAR measures the average activity of the osteoblasts in a section of bone being analyzed. The results of this study show that minocycline treatment increases the area of bone surface actively mineralizing and the overall rate of new bone formation. These outcomes hold promise, since evidence of enhanced mineralization means the organism may be able to counter the early deleterious effects of SCI on bone integrity. The extent of bone loss in individuals suffering with SCI is thought to be multifactorial, being influenced by the degree of injury, extent of injury (complete or incomplete), age, and length of time since injury (Jones et al. 2002, Maynard et al. 1995). Given the age of our subjects (roughly three months), rescuing bone formation is clinically important.

In spite of the vehicle-treated SCI group having higher total BMD, which is a composite of cortical and cancellous measures, Figures 6 and 7 show that the vehicle-treated SCI subjects had higher cortical BMD but slightly lower cancellous BMD compared to the minocycline group. Cortical bone is compact, consisting of closely packed haversian systems; it is much denser, stronger, and stiffer than its cancellous counterpart. Cancellous bone is spongy, has a greater surface area, and is ideal for metabolic activity, such as exchange of calcium ions. Thus, even though the vehicle-treated SCI group displayed higher total BMD, it may be because the minocycline group had a larger percentage of bone undergoing mineralization in which the bone has not hardened yet, reducing the density measure. To confirm this notion, additional studies would be needed to follow the animals on a longer timeline. Another thing to consider is that, like most other tetracycline drugs, minocycline has been associated with metal ion chelation (Garrido-Mesa et al. 2013). It's possible that minocycline chelates calcium ions and prevents them from being used to densify bones, thus contributing to the lower total BMD seen in Figure 5. It's also possible that calcium ion chelation is what enables minocycline to enhance mineralization, which may eventually contribute substantially to bone density if given enough time. Interestingly, Figures 5 and 7 show that the vehicle-treated SCI group has higher total BMD and cortical BMD compared to the intact group. This could mean that traumatic injury results in some type of compensation through bone calcification, or hardening of the bones. Consistent with this notion, is that intact controls had higher cancellous BMD than the vehicle-treated SCI group in Figure 6.

In summary, administration of minocycline was able to significantly enhance active bone mineralization and overall new bone formation rates in a rodent SCI model compared to vehicle-treated SCI controls. Due to the rapid and severe bone loss observed after SCI, a means to bolster bone quality in individuals suffering with SCI is urgent. The current study suggests that minocycline may be able to significantly remineralize bone after injury, however more exploration should be done in the context of drug-assisted bone remodeling for traumatic injury recovery. The results of this study are promising, but would be more reliable if the sample size per treatment group were increased. Studying older animals, where density is the primary outcome measure, may also provide insight into the drug's effects in more mature populations. Additionally, following subjects for an elongated period of time may shed some light on the long-term effects of the augmented mineralization induced by minocycline to see whether it will eventually do significant work on cortical bone as well. Serum assessments could also be incorporated to track changes in various cytokines throughout the recovery period to see what correlation there may be between minocycline treatment and osteoclastogenesis. To deepen the understanding of the effects of calcium ion chelation in post-SCI bone loss, an alternative treatment using a non-chelating tetracycline such as 12S-hydroxy-1,12-pyrazolinominocycline could be incorporated as a control group. With the data imparted from this study, minocycline continues to bear promise in the field of post-injury osteology. Even though it may not reduce bone resorption, it seems to be actively supporting new bone formation, which could be just as important in maintaining bone integrity. Due to the complexity of neurogenic osteoporosis, which describes bone deterioration after SCI (Weiss 2008), increased research is essential for the benefit of the spinally injured population.

REFERENCES

1. **Donnelly DJ, Popovich PG.** 2008. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. *Exp Neurol* 209:378-388.
2. **Perez-Castrillon JL, Olmos JM, Gomez JJ, Barrallo A, Riancho JA, Perera L, Valero C, Amado JA, Gonzalez-Macias J.** 2000. Expression of opioid receptors in osteoblast-like MG-63 cells, and effects of different opioid agonists on alkaline phosphatase and osteocalcin secretion by these cells. *Neuroendocrinology* 72:187-194.
3. **Schett G.** 2011. Effects of inflammatory and anti-inflammatory cytokines on the bone. *Eur J Clin Invest* 41:1361-1366.
4. **Kobayashi K, Imagama S, Ohgomori T, Hirano K, Uchimura K, Sakamoto K, Hirakawa A, Takeuchi H, Suzumura A, Ishiguro N, Kadomatsu K.** 2013. Minocycline selectively inhibits M1 polarization of microglia. *Cell Death Dis* 4:e525.
5. **Boskey AL, Spevak L, Paschalis E, Doty SB, McKee MD.** 2002. Osteopontin deficiency increases mineral content and mineral crystallinity in mouse bone. *Calcified Tissue International* 71:145–154.
6. **Longhini R, de Oliveira PA, Sasso-Cerri E, Cerri PS.** 2014. Cimetidine reduces alveolar bone loss in induced periodontitis in rat molars. *Journal of Periodontology* 85:1115–1125.
7. **Sobacchi C, Schulz A, Coxon FP, Villa A, Helfrich MH.** 2013. Osteopetrosis: genetics, treatment and new insights into osteoclast function. *Nature Reviews Endocrinology* 9:522–536.
8. **Gilbert SF.** 2000. Osteogenesis: The Development of Bones. *In* *Developmental Biology* 6th ed. [Online.] Sinauer Associates, Sunderland, MA. <http://www.ncbi.nlm.nih.gov/books/NBK10056/>
9. **Florencio-Silva R, Sasso GR, Sasso-Cerri E, Simoes Mj, Cerri PS.** 2015. Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. *Boomed Res Int.* doi: 10.1155/2015/421746.
10. **Kornak U, Kasper D, Bosl MR.** 2001. Loss of the CIC-7 chloride channel leads to osteopetrosis in mice and man. *Cell* 104:205–215.

11. **Graves AR, Curran PK, Smith CL, Mindell JA.** 2008. The Cl⁻/H⁺ antiporter ClC-7 is the primary chloride permeation pathway in lysosomes. *Nature* 453:788–792.
12. **Turk N, Cukovic-Cavka S, Korsic M, Turk Z, Vucelic B.** 2009. Proinflammatory cytokines and receptor activator of nuclear factor kappaB-ligand/osteoprotegerin associated with bone deterioration in patients with Crohn's disease. *Eur J Gastroenterol Hepatol* 21:159-166.
13. **Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ.** 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93:165-176.
14. **Souza PP, Lerner UH.** 2013. The role of cytokines in inflammatory bone loss. *Immunol Invest* 42:555-622.
15. **Malec J, Neimeyer R.** 1983. Psychologic prediction of duration of inpatient spinal cord injury rehabilitation and performance of self-care. *Arch Phys Med Rehabil* 64:359-363.
16. **Kurtulus K, Canan A, Sumru O, Nilufer K, Ordu G.** 2006. Evaluation of Bone Mineral Density in Patients with Spinal Cord Injury. *J Spinal Cord Med.* 29(4): 369-401.
17. **Lin T, Tong W, Chandra A, Hsu S, Jia H, Zhu J, Tseng W, Levine MA, Zhang Y, Yan S, Liu XS, Sun D, Young W, Qin L.** 2015. A comprehensive study of long-term skeletal changes after spinal cord injury in adult rats. *Bone Research.* doi:10.1038/boneres.2015.28.
18. **Basso DM, Beattie MS, Bresnahan JC.** 1995. A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 12:1-21.
19. **Dempster DW, Compston JE, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR, Parfitt AM.** 2013. Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 28:2-17.
20. **Schell H, Lienau J, Epari DR, Seebeck P, Exner C, Muchow S, Bragulla H, Haas NP, Duda GN.** 2006. Osteoclastic activity begins early and increases over the course of bone healing. *Bone.* 38(4):547-54.
21. **Mansour A, Abou-Ezzi G, Sitnicka E, Jacobsen SE, Wakkach A, Blin-Wakkach C.** 2012. Osteoclasts promote the formation of hematopoietic stem cell niches in the bone marrow. *J Exp Med.* 209(3):537-49. doi: 10.1084/jem.20110994.

22. **Weitzmann MN.** 2013. The Role of Inflammatory Cytokines, the RANKL/OPG Axis, and the Immunoskeletal Interface in Physiological Bone Turnover and Osteoporosis. Scientifica. <http://dx.doi.org/10.1155/2013/125705>.
23. **Brewer KL, Bethea JR, Yeziarski RP.** 1999. Neuroprotective effects of interleukin-10 following excitotoxic spinal cord injury. *Emergency Medicine*. 159(2):484-493.
24. **Hook MA, Washburn SN, Moreno G, Woller SA, Puga A, Lee KH, Grau JW.** 2011. An IL-1 receptor antagonist blocks a morphine-induced attenuation of locomotor recovery after spinal cord injury. *Brain Behav Immun*. 25(2):349-59. doi: 10.1016/j.bbi.2010.10.018.
25. **Shadid CS, Barrett JR.** 9 October 2013, posting date. Opioid-induced Osteoporosis: Assessing Causes and Treatments. *Practical Pain Management*. 13:10. [Online.] <http://www.practicalpainmanagement.com/treatments/pharmacological/opioids/opioid-induced-osteoporosis-assessing-causes-treatments>.
26. **Brennan MJ.** 2013. The effect of opioid therapy on endocrine function. *Am J Med*. 126(3): 512-18.
27. **Smith H, Elliott J.** 2012. Opioid-induced Androgen Deficiency (OPIAD). *Pain Physician*. 15:ES145-ES156.
28. **Zhang JM, An J.** 2009. Cytokine, inflammation and pain. *Int Anesthesiol Clin*. 45(2): 27-37. doi:10.1097/AIA.0b013e318034194e.
29. **Watkins LR, Maier SF.** 2002. Beyond neurons: Evidence that immune and glial cells contribute to pathological pain states. *Physiological Reviews*. 82:981-1011.
30. **Wells JE, Hurlbert RJ, Fehlings MG, Yong VW.** 2003. Neuroprotection by minocycline facilitates significant recovery from spinal cord injury in mice. *Brain*. 126(7):1628-37.
31. **Nicholson B, Verma S.** 2004. Comorbidities in chronic neuropathic pain. *Pain Med*. 5(1):S9-S27.
32. **Garrido-Mesa N, Zarzuelo A, Galvez J.** 2013. Minocycline: far beyond an antibiotic. *Br J Pharmacol*. 169(2): 337-352. doi: 10.1111/bph.12139.
33. **Duarte RV, Raphael JH, Southall JL, Labib MH, Whallett AJ, Ashford RL.** 2013. Hypogonadism and low bone mineral density in patients on long-term intrathecal opioid delivery therapy. *BMJ Open*. doi: 10.1136/bmjopen-2013-002856.

34. **Dionyssiatis Y, Lyritis GP, Mavrogenis AF, Papagelopoulos PJ.** 2011. Factors influencing bone loss in paraplegia. *Hippokratia*. 15:54-59.
35. **Jones LM, Legge M, Goulding A.** 2002. Intensive exercise may preserve bone mass of the upper limbs in spinal cord injured males but does not retard demineralization of the lower body. *Spinal Cord*. 40:230-235.
36. **Szollar SM, Martin EM, Sartoris DJ.** 1998. Bone Mineral Density and Indexes of Bone Metabolism in Spinal Cord Injury. *Am Journal of Physic Med Rehabil*. 77:28-35.
37. **Wilmet E, Ismail A, Heilporn A.** 1995. Longitudinal study of bone mineral content and of soft tissue composition after spinal cord section. *Paraplegia*. 33(11):674-677.
38. **Garland DE, Stewart CA, Adkins EH.** 1992. Osteoporosis after spinal cord injury. *Journal of Orthop Res and Dev*. 45(2):283-296.
39. **Eser P, Frotzler A, Zehnder Y.** 2004. Relationships between the duration of paralysis and bone structure: A pQCT study of spinal cord injured individuals. *Bone*. 34(5):869-880.
40. **Sorensen FB, Bohr H, Schaadt O.** 1998. Bone mineral content of the lumbar spine and lower extremities years after spinal cord lesion. *Paraplegia*. 26:293-301.
41. **Shields RK.** 2002. Muscular, Skeletal, and Neural adaptations Following Spinal Cord Injury. *J of Orthopaedic and Sports Phys Therapy*. 32(2):65-74.
42. **Rittweger J, Jerris K, Altenburg T.** 2006. Bone adaptation to altered loading after spinal cord injury: a study of bone and muscle strength. *Journal of Musculoskeletal Neuronal Interact*. 6(3):269-276.
43. **Giangregorio L, McCertney N.** 2006. Bone loss and muscle atrophy in spinal cord injury: epidemiology, fracture prediction, rehabilitation strategies. *J Spinal Cor Med*. 29(5):489-500.
44. **Hangartner TN, Rodgers MM, Glaser RM.** 1994. Tibial bone density loss in spinal cord injured patients: effects of FES exercise. *Journal of Rehabil Res Dev*. 31:50-61.
45. **Svircev J.** 2007. Osteoporosis and spinal cord injury. SCI Forum Report. Presented October 9, 2007. <http://sci.washington.edu/info/forums/reports/oestoporosis.asp> Retrieved 6/11/2010
46. **Frost HM.** 1985. The new bone: some anthropological potentials. *Yearbook Phys Anthropol*. 28:211-226.

47. **Frey-Rindova P, de Bruin ED, Stussi E.** 2000. Bone mineral density in upper and lower extremities during 12 months after spinal cord injury measured by peripheral quantitative computed tomography. *Spinal Cord.* 38:36-32.
48. **Maynard FM, Karunas RS, Adkins RH.** 1995. Management of the Neuromuscular System., in *Spinal Cord Injury: Clinical Outcomes of the Model Systems*, SL Stover, JA Delisa, and GG Whitenecks, es, Aspen, Gaithersburg, MD. pp. 163-169.
49. **Weiss D.** 2008. Osteoporosis and Spinal Cord Injury. <http://emedicine.medscape.com/article/322204-overview>